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Transgenic mice expressing the activated neu oncogene in the mammary epithelium develop mammary tumors with relatively short latency. To explore the role of the EGFR in tumorigenesis in this system we have recently crossed these MMTV/activated neu strains to a naturally occuring mouse mutant which expresses a catalytically inactive EGFR (Waved-2 strain). The results of these analyses revealed that the catalytic activity of the EGFR is dispensible for neu-induced mammary tumorigenesis. These observations suggest that the catalytic activity of EGFR is dispensible for neu-mediated tumorigenesis. In addition to these studies, we have also examined the spectrum of EGFR receptor family in the activated neu induced mammary tumors. The results of these analyses revealed that in addition to Neu expression, erbB-3 expression could be detected at high levels. These observations suggest that the erbB-3 and Neu act cooperatively to induce mammary tumors.

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EXPRESSION OF THE EPIDERMAL GROWTH FACTOR RECEPTOR FAMILY IN TRANSGENIC MOUSE MODELS OF HUMAN BREAST CANCER.

INTRODUCTION:

The Epidermal Growth Factor receptor family comprises four closely related type 1 receptor tyrosine kinases (RTK's) (EGFR, Neu [erbB-2, HER2], erbB-3 [HER3], erbB-4 [HER4] that a receptors for a variety of mitogenic growth factors (Ullrich and Schlessinger, 1990). Elevated expression of several of these EGFR family members has been implicated in the genesis of human breast cancer. For example, amplification and consequent overexpression of Neu has been observed in a significant proportion of human breast cancer (King et al., 1985, Slamon et al., 1987, Slamon et al., 1989). Moreover the extent of Neu overexpression has also been inversely correlated with patient survival (Paterson et al., 1991, Gullick et al., 1991). More recently, elevated expression of other members of the EGFR family including EGFR, erbB-3 and erbB-4 have been noted in both primary breast tumors and their derived cell lines (Lacroix et al., 1988, Kraus et al., 1989, Plowman et al., 1990, Plowman et al., 1990).

Whereas elevated expression of these EGFR family members have been detected in human breast cancer, there is also evidence to suggest that the activity of these receptors can be influenced by expression of EGFR specific ligands. For example, the expression of EGFR family ligands such as TGFα, EGF, and NDFs (Neu differentiation factors) have been implicated in the pathogenesis of human breast cancer (Salomon et al., 1990). Although the Neu cannot directly interact with these ligands, its tyrosine kinase activity can be profoundly influenced by expression of the EGFR family ligands. Indeed, Neu is the substrate for the activated EGFR following stimulation of cells with EGF or TGFα (Stern and Kamps, 1988, Kokai et al., 1988, Goldman et al. 1990). Similarly, Neu can be transphosphorylated by either erbB-3 and erbB-4 following stimulation of cells with the NDFs (Karunagaran et al., 1996). The ability of these growth factors to modulate the activity of Neu is thought to mediated through the formation of specific heterodimers of Neu and the different EGFR family members (Wada et al.,1990). Consistent with these observations, co-expression of Neu and EGFR or Neu and ErbB-3 results in the efficient transformation of fibroblasts in vitro (Kokai et al., 1989).

More recently, it has been demonstrated that retention of Neu in the endoplasmic reticulum, through the expression of the Neu specific single chain antibody, can interfere with ability of breast cancer cells to respond to the mitogenic stimulation of both EGF and NDFs (Graus-Porta et al. 1997). The central importance of Neu in signaling by EGF or NDF is further highlighted by the observation that heterodimers between EGFR and Neu or erbB-3/erbB-4 and Neu results in the induction of high affinity receptors for these potent growth factors (Karunagaran et al., 1996, Wada et al., 1990). Conversely, expression of the the Neu ecto domain lacking catalytic activity can interfere with the ability of the activity of the EGFR to transform glial cells (Rourke et al., 1997). Taken together, these observations suggest that the interaction of these different EGFR family members play an important role in tumor progression.

Direct evidence for the importance of these EGFR family members and their cognate ligands in the induction of mammary tumors derives from studies of transgenic mice that have been egineered to express elevated levels of these genes in the mammary epithelium. For example, elevated expression of a constitutvely active form of Neu resulted in the rapid induction of multifocal mammary tumors within a short latency period (Muller et al., 1988). Mammary epithelial specific expression of the wild type neu proto-oncogene also resulted in the induction of mammary tumors (Guy et al., 1992). However in contrast to the rapid development of mammary tumor observed in mice expressing the constitutively active Neu isoform, the tumors arising in the wild type strains were focal in origin. Significantly, the induction of mammary tumors in the wild-type neu miice correlated in a majority of tumor samples with the occurrence of activating mutations in the transgene (Siegel et al., 1994, Siegel and Muller, 1996). Molecular analyses has revealed that oncogenic conversion of Neu in these strains involves mutations which promote receptor dimerization through the formation of cysteine disulfide bonds (Siegel et al., 1996). These

observations suggest that the catalytic activation of Neu is critical for the induction of mammary carcinoma.

Additional evidence supporting the role of the EGFR family in mammary tumorigenesis derives from observations with transgenic mice expressing several EGFR ligands. Transgenic mice expressing TGFα in the mammary epithelium develop global mammary epithelial hyperplasias which occcaisionally progress to overt mammary carcinomas (Matsui et al., 1989, Jhapppan et al., 1989, Sandgren et al., 1989). More recently, mammary epithelial expression of NDF has been reported to result in the induction of mammary tumors (Krane and Leder, 1996). However, like the MMTV/wild type neu transgenic strains, the induction of mammary tumors in these strains appears to require additional genetic events. The activity of the EGFR receptor family has also been implicated a important factor in normal mammary gland development. For example, a naturally occuring mutation in the EGFR which results in the severe impairment of kinase activity (Waved-2 mice) leads to a dramatic lactation defect (Lutteke et al., 1994, Fowler et al., 1995).

The primary purpose of our Army-sponsored research program has been to elucidate the role of the various EGFR family members in mammary tumorigensis. Our initial efforts focused on the role of the EGFR in Neu- mediated tumorigenesis. We have previously demonstrated that coexpression of TGFα and Neu in the mammary epithelium resulted in the development of mammary tumors within a significantly shorter period than transgenic mice expressing either Neu or TGFα alone in the mammary epithelium (Muller et al., 1996: see previous annual report). Conversely, we have also shown that catalytic activity of the EGFR is dispensible for the induction of tumors by Neu since homozygous Waved-2 mice expressing Neu in the mammary epithelium develop mammary tumors at comparable rates (Kannan, Co and Muller, Figure 1, see Results section)

The primary focus of our research over the last year has been to elucidate the role of erbB-3 and erbB-4 type 1 RTKs in Neu-mediated tumorigenesis. As a first step we examined the expression of the various EGFR family member in transgenic mice expressing an activated Neu oncogene. The results of these analyses revealed that the Neu induced mammary tumors the primary EGFR family that is expressed in these mammary tumors is erbB-3. Although both EGFR and erbB-4 are also expressed in these tumors they are expressed at much lower levels.Unlike EGFR and erbB-4, erbB-3 is also tyrosine phosphorylated (see Results section). Moreover, erbB-3 and Neu are frequently coexpressed in human breast cancers (see Results section).

To further examine the relationship between erbB-3/Neu coexpression in the the induction of mammary tumors, we are currently in the process of deriving transgenic mice expressing a MMTV/erbB-3 fusion gene with the ultimate goal of interbreeding these strains

RESULTS AND DISCUSSION

Expression of activated version of erbB-2 is capable of inducing mammary tumors in the absence of a functional EGFR.

To determine whether the catalytic function of the EGFR was absolutely required for the induction of mammary tumors, we have crossed a natural occuring mouse mutation lacking a catalytic function of the EGFR with transgenic mice expressing an activated form of the erbB-2 RTK in the mammary epithelium (NDL 1-2 strains). Because of a deletion in the extracellular domain of neu that leads to receptor dimerization, female mice from this strain reproducibly develop mammary tumors at approximately five months of age. The results of these analyses revealed that incidence of mammary tumors in animals in a waved-2 homozygous background were indistinguishable from those observed in animals either heterozygous for the waved-2 mutation or wild type genotype (Figure 1). One complication with these obsevations is that relative to original tumor kinetics observed with the activated neu strains tumors in both Waved-2 mutation and wild type background occured with considerably longer latency and lower penetrance. This delay of tumor formation is likely due to the fact that the influence of different genetic background on the expression levels of the activated neu transgene since RNase protection analyses on RNA samples from the different normal mammary epitheliual samples failed to reveal the presence of the

activated neu transcript. However, in mammary tumors that arise in these Waved-2/activated neu transgenic, abundant neu transcripts can be detected (see previous annual report).

Although the results of this cross suggest that Neu-induced mammary tumors can occur in the Waved-2 background, the observed heterogeniety of neu transgene expression observed in these crosses complicates the interpretation on whether the efficiency of induction of mammary tumors is influenced by the Waved-2 mutation. To address this potential concern, we have examined the effect of genetic background on transgene expression in different transgenic strains carrying a different lower transforming alleles of neu (the NDL2-5 strains). By contrast to the initial NDL 1-2 strains where neu transgene transcript could not be detected in normal mammary epithelium on the Waved-2 genetic background, abundant NDL 2-5 transcripts could be detected in the mammary tissue in this background (unpublished observations). Based on these observations, we are currently interbreeding the NDL 2-5 strains to the Waved-2 mice to confirm the observations made the NDL-1-2/Waved -2 crosses.

In addition, to the continued phenotypic characterization of these strains, we also plan to perform detailed biochemical characterization of the EGFR, erbB-2, erbB-3 and erbB-4 in these mammary tumors. In particular, we plan to measure the state of tyrosine phosphorylation of either the EGFR family in tumors derived from the various NDL/Waved-2 geneotypes by performing immunoprecipitation/immunoblot analyses with EGFR family specific antibodies and phophotyrosine specific antisera. In addition, we will also use this immunopreciptation/immunoblot approach to assess whether erbB-2 protein in these tumors can be detected as either homodimers or heterodimers with other members of the EGFR family . The results of these additional genetic and biochemical analyses will provide important insight into the role of the EGFR and erbB-2 protein in the induction of mammary tumors.

Expression of activated forms of Neu/erbB-2 oncogene in transgenic mice results in the coordinate upregulation and activation of erbB-3 during mammary tumorigenesis.

Whereas activation of the EGFR appears to be dispensible for the induction of mammary tumors in the activated neu strains, we have recently derived evidence that elevated expression of the erbB-3 may be implicated in Neu mediated tumorigenesis. To explore the potential role of EGFR family members in tumors arising in the activated neu transgenic strains (NDL strains), we performed immunoblot analyses on tumor extracts derived from the activated neu transgenic strains with antibodies specific to the various EGFR family members. The results of these analyses revealed that in addition to elevated levels of erbB-2, the mammary tumors derived from these various NDL strains consistently expressed elevated levels of erbB-3 (Figure 2). By comparison, the levels of either EGFR or erbB-4 were considerably lower (Figure 2). To further confirm that observed elevated levels of erbB-3 expression were functionally important in the induction of tumors by neu we also determined whether erbB-3 in these tumors is tyrosine phosphorylated by performing immunoblot/immunoprecipitation analyses on the tumor extracts with erbB-3 and antiphoshotyrosine specific antisera. The results of these experiments revealed that like the activated erbB-2 protein, the erbB-3 protein was tyrosine phosphorylated in these tumors (Figure 3). Because erbB-3 does not appear to possess a catalyticly active tyrosine kinase (Guy et al., 1994), the observed tyrosine phosphorylation of erbB-3 is likely due to transphosphorylation of erbB-3 by the activated neu transgene.

Given the apparent coordinate regulation of erbB-2 and erbB-3 expression during mammary tumor progression in these strains, we plan to further determine whether we can detect heterodimers of erbB-2 and erbB-3 in these tumors. To accomplish this, we plan to initially use immunoblot/immunoprecipitation analyses with erbB-2 and erbB-3 specific antisera. These analyses should provide evidence that the observed tyrosine phosphorylation on erbB-3 is due to its interaction with erbB-2. However given the transient nature of interaction between heterodimers of the EGFR family, it may be necessary to crosslink these heterodimers with chemical crosslinking agent to confirm the presence of these erbB-2/erbB-3 heterodimers.

In addition to these biochemical analyses, we plan to further explore the functional significance of erbB-3 in erbB-2 mediated mammary tumorigenesis. One potential means of addressing the functional importance of the erbB-3 in mammary tumorigenesis is to generate transgenic strains expressing erbB-3 in the mammary epithelium. To this end, we have recently generated several independent transgenic strains that carry a MMTV/erbB-3 fusion gene (Figure 4).Because we have just recently derived the strains, the bulk of the remaining year of Army support will be devoted to the detailed morphological and biochemical characterization of these MMTV/erbB-3 strains. These analyses will include a RNase protection analyses of the tissue specific pattern expression of the transgene. In addition, we plan to assess the levels of erbB-3 protein expression in these tissues. After the completion of the characterization of these strains at the end of the funding period, we will select appropriate strains that expresss elevated levels of erbB-3 for interbrredd experiments with the MMTV/wild type neu strains (Guy et al., 1992).

Another important functional aspect of erbB-3's role in erbB-2 mediated transformation is it thought to couple the phosphadityl inositol 3' kinase (PI-3' kinase) signaling pathway to the erbB-2 receptor (Pringent and Gullick, 1994: Sotloff et al., 1994). To explore the importance of this signaling pathway in erbB-2 mediated mammary tumorigensis we have constructed an inducible dominant negative PI-3' kinase inhibitor expression cassette. The basis of the dominant negative action of the mutant PI-3' kinase derives from specific mutation in the SH2 bearing p85 subunit which prevents its association with the 110 kDa catalytic subunit. As a consequence of this mutation this dominant negative inhibitor can occupy its binding site but is catalytically inert (Kotani, 1994). Because stable of expression of this dominant negative inhibitor of the PI-3' kinase appears not to be compatible with cell viability we have placed a pgk-neo transcription unit as a transcriptional stop sequence flanked by LOX recombination sites between the MoMuLV promoter and the cDNA encoding the mutant p85 cDNA (Figure 5). By transiently expressing the Cre recombinase in cells carrying this construct the LOX flanked pgk-neo cassette can be excised leading to the induction of expression of the PI-3' kinase dominant inhibitor. To assess whether expression of this dominant negative inhibitor of the PI-3' kinase could interfere with erbB-2 mediated transformation, we plan to derive stable mammary tumor cells lines coexpressing erbB-2 and erbB-3 (derived from the MMTV/activated neu strains) that carry the inducible PI-3' kinase inhibitor. To induce the expression of the PI-3' kinase dominant negative inhibitor, mammary tumor cell lines expressing both erbB-2 and erbB-3 will transiently transfected with Cre recombinase expression plasmids or use Adenoviral based Cre expression systems. We will then assess if induction of the PI-3' kinase inhibitor effects the transformation properties of these mammary tumor cells. To assess the ability of the dominant negative inhibitor to influence the transformation properties of these induced cell lines, we plan to test the ability of these cells to grow in soft agar.

One possible outcome of these studies is that induction of PI-3' kinase dominant negative inhibitor may result in the induction of apoptotic cell death. Indeed, there is a increasing body of evidence to suggest that activation of the PI-3' kinase signalling pathway is involved in the regulation of apoptotic cell death in a number of different cell types. For example, abrogation of growth factor-mediated activation of the PI-3' kinase signaling pathway through adminstration of specific PI-3' kinase inhibitors or expression of mutant growth factor receptors decoupled from the PI-3' kinase in a number of cell types results in the induction of apoptotic cell death (Yao and Cooper, 1995). To explore this possibility, we will also plan to subject Cre transfected mammary tumor cell lines to in situ apoptosis assay (TUNEL assay) to assess whether induction of expression of the dominant negative inhibitor of the PI-3' kinase is associated with apoptotic cell death in these cell lines. Indeed, we have used a similar approach to demonstrate that activation of the PI-3' kinase is required to provide an important survival signal in Polyomavirus-mediated mammary tumorigenesis (Webster et al., 1997). The results of these studies should provide important insight into whether the elevated expression of erbB-3 observed in these tumors is involved in coupling to a PI-3' coupled cell survival signalling pathway.

The role of erbB-4 in the induction of mammary tumors in transgenic mice expressing either the activated erbB-2 or neu differention factor (NDF) in the mammary epithelium.

In our previous anual report we reported on the derivation of transgenic mice expressing the erbB-4 in the mammary epithelium (see previous annual report). By contrast to transgenic mice expressing the wild type erbB-2 which develop solitary mammary tumors after long latency, none of the MMTV/erbB-4 mice have developed mammary tumors. To further explore the role of erbB-4 in mammary tumorigenesis we have interbreed selected strains of the MMTV/erbB-4 strains with either the MMTV/activated erbB-2 strains (NDL2-5) or the MMMTV/NDF strains (Krane et al., 1996). To date we have generated 10 erbB-4/activated neu and 3 erbB-4/NDF bigenic females with similar numers of monogenic control animals. Because we are currently only four months into the analyses it is too early to asess whether coexpression of erbB-4 with either activated erbB-2 or NDF will influence the kinetics of development of mammary tumors. One possible outcome of these studies is that coexpression of either NDF or activated erbB-2 with erbB-4 will result in a dramatic acceleration of mammary tumors in bigenic animals as compared to the monogenic animals alone. However it is also possible that the interbreeding of the erbB-4 with these strains may interfere with tumor development in these strains. Indeed, there is evidence to suggst that elevated expression of erbB-4 may in fact signal an antipoliferative signal in the mammary epithelium (David Stern, personal communication). Whatever the outcome of these studies, we will also perform biochemical analyses on the mammary epithelium from the progeny of these crossess. In particular we plan to determine the levels both erbB-2 and erbB-4 in both normal and tumor epithelium by performing immunoblot analyses with erbB-2 and erbB-4 specific antisera. In addition, we plan to assess the state of tyrosine phosphorylation of erbB-2 and erbB-4 in these tissues by performing immunoprecipitation and immunoblot analyses with erbB-2 , erbB-4 and antiphotyrosine antibodies. These biochemical analyses should compliment the results of the genetic crosses. Finally we also plan to initiate interbreedings between the MMTV/erbB-3 and MMTV/erbB-4 transgenic strains to assess whether coexpression of these two EGFR family members can cooperate to accelerate mammary tumorigenesis. The characterization of these interbreedings will proceed as outlined for the interbreedings of the other EGFR expressing strains. At the completion of the final year of Army sponsored funding we should have gained important insigth into the role these various EGFR family members play in mammary tumorigensis.

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APPENDIX 1

FIGURES 1-5

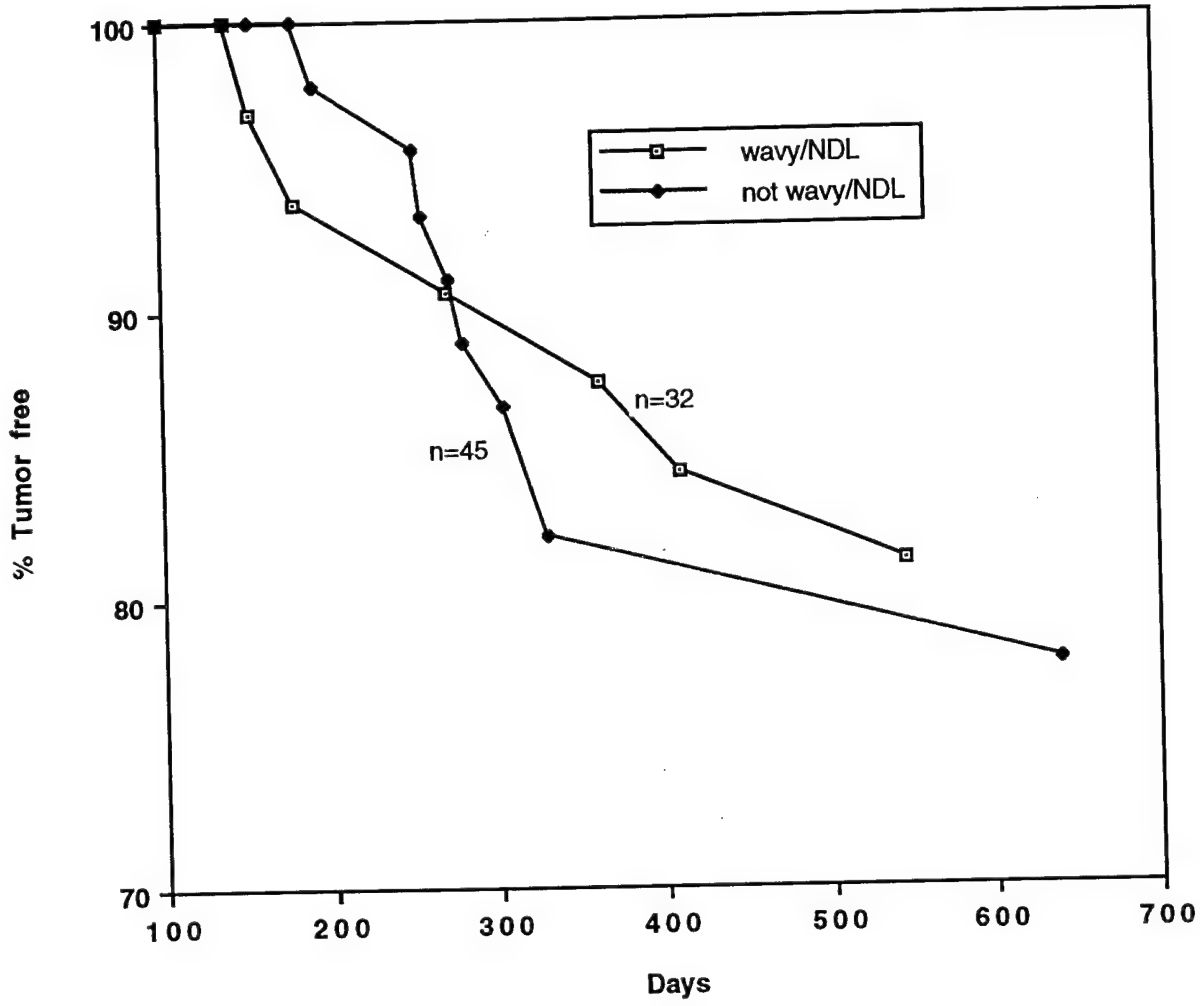


Fig.1: Mammary tumor onset in wavy/NDL and not wavy/NDL mice

FIG. 2. The expression of endogenous ErbB-3 receptors is significantly increased, relative to that of the EGFR and ErbB-4, in tumors derived from both NDL1 and NDL2 transgenic animals. (A) Equivalent amounts of total protein (50 μg) obtained from mammary tumor lysates (BT) were electrophoresed on an SDS-4 to 12% polyacrylamide gradient gel and transferred to a PVDF membrane (lanes 2 to 9). The membrane was cut, and the upper half of the blot was probed with EGFR-specific mouse monoclonal antibodies while the lower portion of the membrane was blotted with Grb-2-specific rabbit polyclonal antisera. Similarly, immunoblots were performed for both ErbB-3 (B) and ErbB-4 (C) using the same tumor lysates. As in (A), Grb-2 immunoblots were used to confirm that equivalent amounts of protein were present in each lane. Lysates from normal, non-transgenic mammary tissue (FVB Mam. Gl.) were included in each panel as a negative control (lane 1).

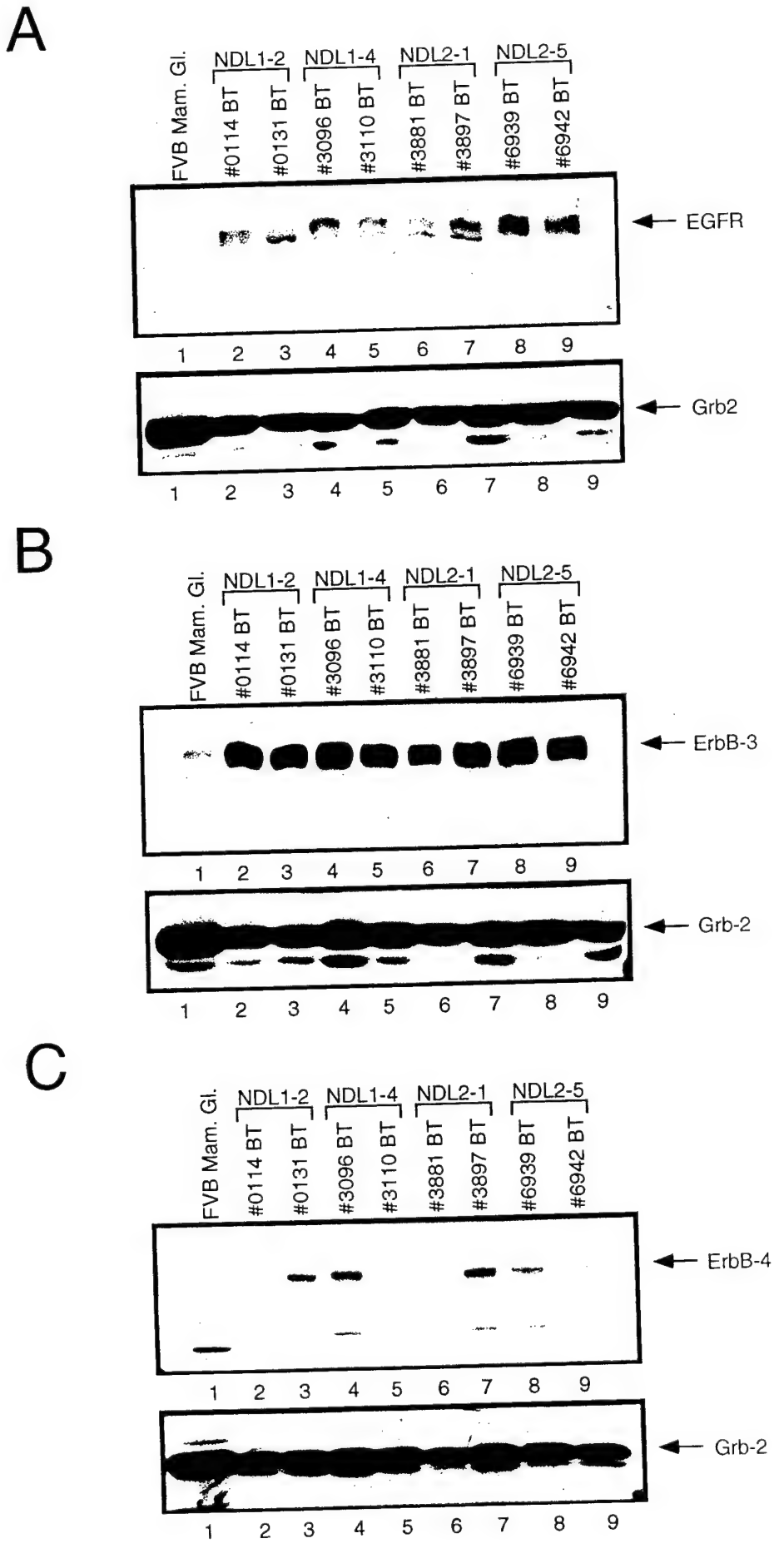
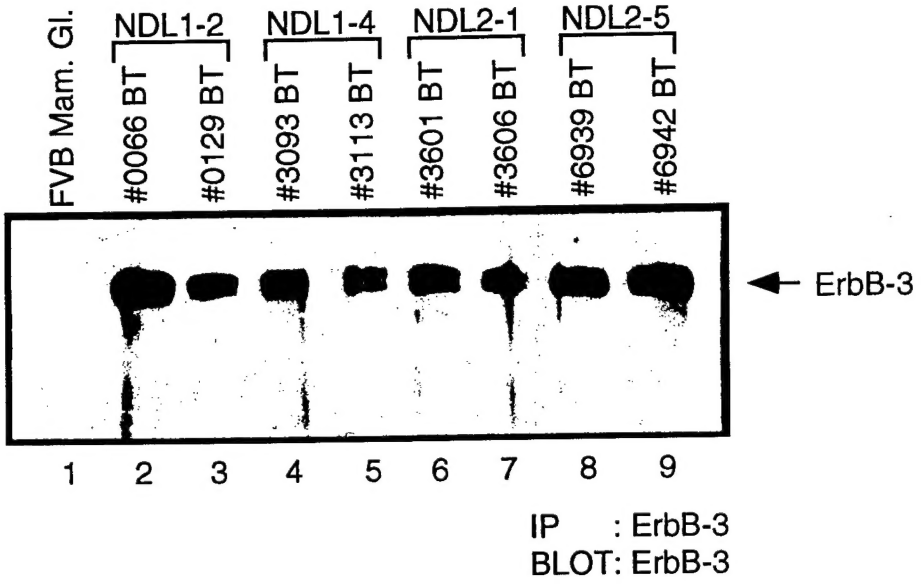

A
FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0114 BT
#0131 BT
#3096 BT
#3110 BT
#3881 BT
#3897 BT
#6939 BT
#6942 BT
EGFR
1 2 3 4 5 6 7 8 9
Grb2
1 2 3 4 5 6 7 8 9
B
FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0114 BT
#0131 BT
#3096 BT
#3110 BT
#3881 BT
#3897 BT
#6939 BT
#6942 BT
ErbB-3
1 2 3 4 5 6 7 8 9
Grb-2
1 2 3 4 5 6 7 8 9
C
FVB Mam. Gl.
NDL1-2
NDL1-4
NDL2-1
NDL2-5
#0114 BT
#0131 BT
#3096 BT
#3110 BT
#3881 BT
#3897 BT
#6939 BT
#6942 BT
ErbB-4
1 2 3 4 5 6 7 8 9
Grb-2
1 2 3 4 5 6 7 8 9

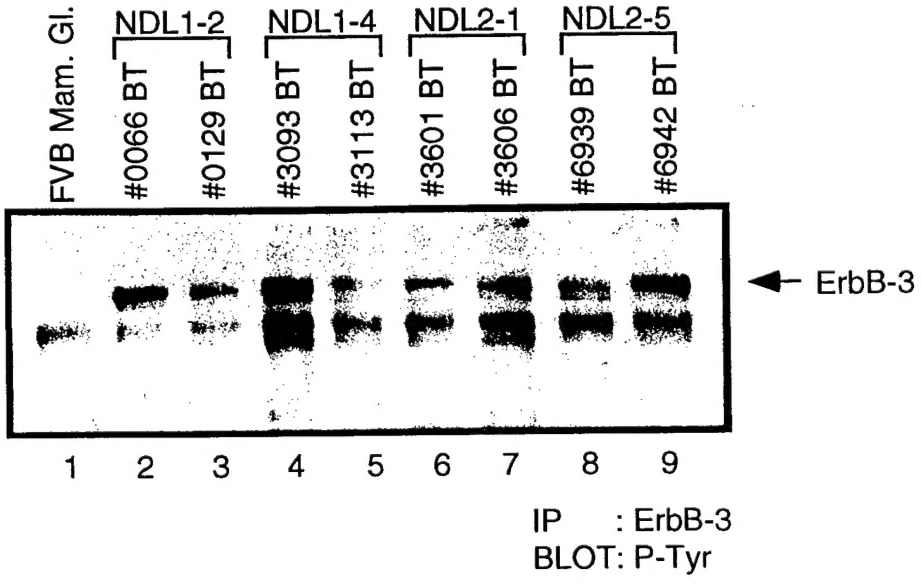
FIG. 3. Endogenously expressed ErbB-3 is tyrosine phosphorylated. (A) ErbB-3 was immunoprecipitated (IP) from mammary tumor lysates (BT) derived from NDL transgenic animals (NDL1 and NDL2). One-third of the immunoprecipitate was electrophoresed on an SDS-9.0% polyacrylamide gel, transferred to a PVDF membrane, and subjected to immunoblot (BLOT) analyses with an ErbB-3 specific antibody (lanes 2 to 9). The position of ErbB-3 is indicated by the arrow. (B) The remaining two-thirds of the immunoprecipitate was subjected to immunoblot analyses with anti-phosphotyrosine (P-Tyr) specific antibodies, as described in (A). The position of tyrosine phosphorylated ErbB-3 is indicated by the arrow. A lysate from a non-transgenic mammary tissue (FVB Mam. Gl.) was included in the IP as a negative control (lane 1).

A



IP : ErbB-3
BLOT: ErbB-3

B



IP : ErbB-3
BLOT: P-Tyr

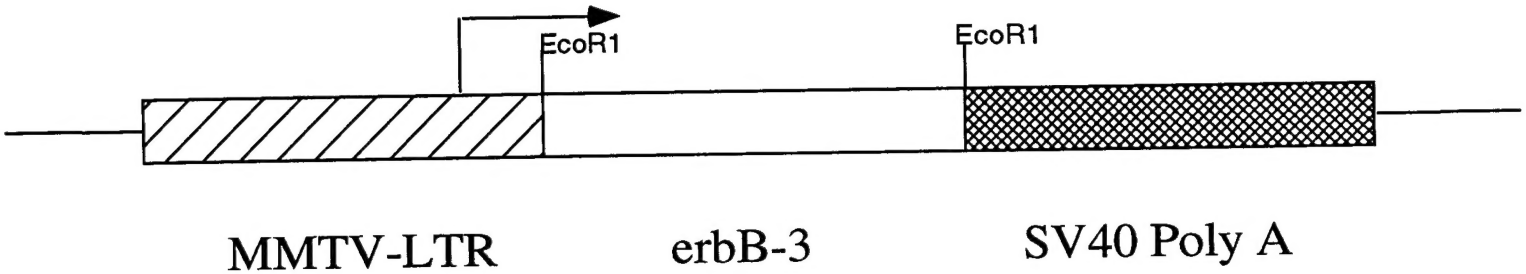


Fig.4:Transgene construct for generating MMTV-erbB3 mice

A

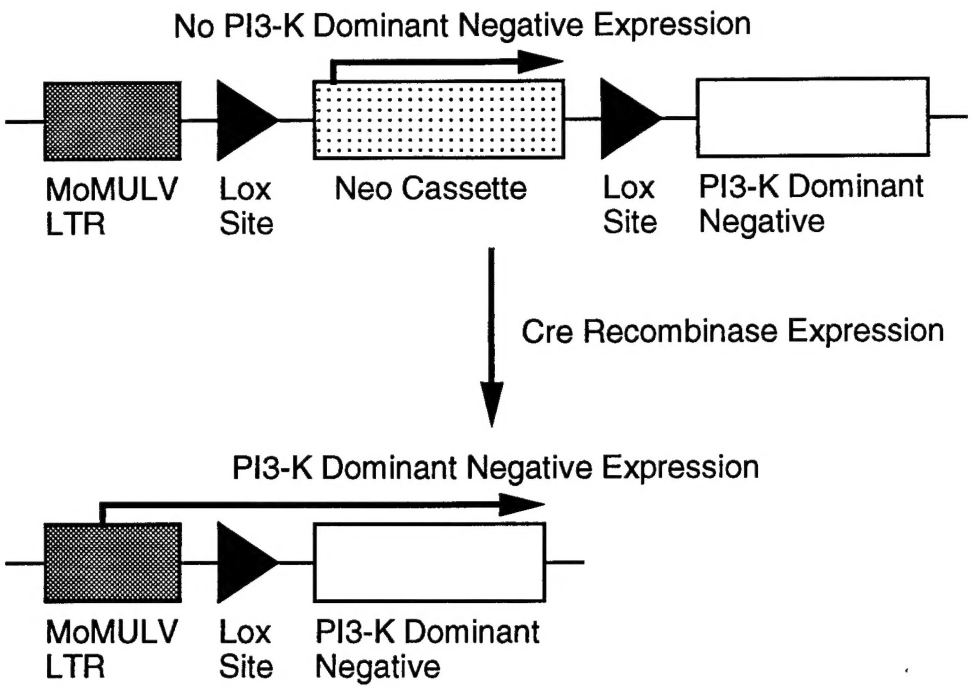

No PI3-K Dominant Negative Expression
MoMULV LTR
Lox Site
Neo Cassette
Lox Site
PI3-K Dominant Negative
Cre Recombinase Expression
PI3-K Dominant Negative Expression
MoMULV LTR
Lox Site
PI3-K Dominant Negative

FIGURE 5